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**Conservation of lipid metabolic gene transcriptional regulatory networks  
in fish and mammals**

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## Abstract

Lipid content and composition in aquafeeds have changed rapidly as a result of the recent drive to replace ecologically limited marine ingredients, fishmeal and fish oil (FO). Terrestrial plant products are the most economic and sustainable alternative; however, plant meals and oils are devoid of physiologically important cholesterol and long-chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic (EPA), docosahexaenoic (DHA) and arachidonic (ARA) acids. Although replacement of dietary FO with vegetable oil (VO) has little effect on growth in Atlantic salmon (*Salmo salar*), several studies have shown major effects on the activity and expression of genes involved in lipid metabolism. In vertebrates, sterols and LC-PUFA play crucial roles in lipid metabolism by direct interaction with lipid-sensing transcription factors (TF) and consequent regulation of target genes. The primary aim of the present study was to elucidate the role of key TFs in the transcriptional regulation of lipid metabolism in fish by transfection and overexpression of TFs. The results show that the expression of genes of LC-PUFA biosynthesis (*elovl* and *fads2*) and cholesterol metabolism (*abca1*) are regulated by Lxr and Srebp TFs in salmon, indicating highly conserved regulatory mechanism across vertebrates. In addition, *srebp1* and *srebp2* mRNA respond to replacement of dietary FO with VO. Thus, Atlantic salmon adjust lipid metabolism in response to dietary lipid composition through the transcriptional regulation of gene expression. It may be possible to further increase efficient and effective use of sustainable alternatives to marine products in aquaculture by considering these important molecular interactions when formulating diets.

## Key words

Atlantic salmon; fatty acid; gene expression; lipid; pyloric caeca; transcription factor

## 1. Introduction

Lipid content and composition in feeds for farmed fish has experienced a recent and rapid change, because, in order to sustain growth of the aquaculture industry, ecologically limited marine fish meal and fish oil (FO) ingredients have been replaced by terrestrial plant-derived meals and oils. Although replacement of up to 100% dietary FO with vegetable oil (VO) has little effect on growth in Atlantic salmon (*Salmo salar*) [1], some studies have shown major effects on the expression and regulation of genes involved in fatty acid and cholesterol metabolism [2,3]. This impact was consistent with the major compositional changes caused by feeding VO to fish including decreased levels of dietary cholesterol and long-chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoate (EPA; 20:5n-3), docosahexaenoate (DHA; 22:6n-3) and arachidonate (ARA, 20:4n-6), which are absent in terrestrial plants [2]. Cholesterol and LC-PUFA are critical functional components of cellular membranes and are important precursors of bioactive lipids required for homeostasis, cell signaling, immune and inflammatory responses [4], and the long-term health and welfare effects of reductions in these essential dietary nutrients in fish, including salmon, are unknown.

Much effort has been directed toward the understanding of effects of dietary imbalances in LC-PUFA and cholesterol in humans and mammalian models because of the links between dyslipidemia and a range of highly prevalent cardiovascular, metabolic and inflammatory diseases [5]. These studies have shown that cholesterol and fatty acids and their metabolic derivatives can exert major effects on physiology by interactions with a range of transcription factors [6]. Particular attention has focused on liver X receptor (LXR), peroxisome proliferator-activated receptors (PPAR) and sterol regulatory element binding proteins (SREBP) and their activities in liver and monocyte cells. LXR has a pivotal role in the control of intermediary metabolism mediating cross-regulation between fatty acid and sterol metabolism [7]. LXR activity is activated by binding oxysterol ligands, catabolic products of cholesterol [8]. In response to cholesterol overloading, and consequent oxysterol production, LXR modulates intracellular cholesterol levels by transactivating the expression of cholesterol ester transfer

protein, apolipoproteins, cholesterol 7 $\alpha$ -hydroxylase (CYP7 $\alpha$ 1) and the ATP-binding cassette transporter 1 (ABCA1), which regulate cholesterol efflux from cells [7].

In response to cholesterol depletion, SREBPs, a family of membrane-bound transcription factors, are activated. SREBP1 plays a crucial role in the regulation of many lipogenic genes and SREBP2 primarily regulates the transcription of cholesterologenic enzymes [9]. Interactions between these pathways are to some extent mediated through LXR activating SREBP1 transcription, inducing the expression of enzymes involved in the synthesis of fatty acids, triacylglycerols and phospholipids. In addition, some important lipid metabolizing genes, such as fatty acid synthase (FAS), are both LXR and SREBP1 targets [10]. PPARs, encoded by three genes in mammals, are activated by binding fatty acids or their oxidized derivatives and act to regulate expression of genes of lipid degradation and biosynthesis. PPAR $\alpha$  and PPAR $\beta$ , regulate the expression of genes encoding mitochondrial and peroxisomal fatty acid-catabolizing enzymes, whilst PPAR $\gamma$  has a central role in fat storage by promoting and maintaining the adipocyte phenotype [6]. Thus, LXR, SREBP and PPAR transcription factors act as lipid sensors that translate changes in cellular sterol and fatty acid content and composition into metabolic activity.

Compared to mammals, few studies have addressed the existence or roles of these transcriptional regulators of lipid metabolism in fish. Our contention is that a greater understanding of lipid-mediated gene regulatory networks in Atlantic salmon will facilitate the efficient, effective and safe use of sustainable alternatives to marine products in aquaculture feeds. Recently the genes for Atlantic salmon Lxr, Srebp1 and Srebp2 and Ppars have been characterized [11-13]. In addition, studies on an Atlantic salmon cell line (SHK-1) have shown that several lipid metabolic genes are transcriptionally regulated in response to changes in lipid composition of the medium [13]. The primary aim of the present study was to elucidate Lxr, Srebp and Ppar gene regulatory mechanisms and key lipid metabolic target genes in Atlantic salmon and to determine the extent to which dietary modulation of lipid and fatty acid metabolism in salmon reflects or varies from the patterns of gene regulation described for mammals.

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## 98 **2. Materials & Methods**

### 99 *2.1. Cell lines and cell culture*

100 The established Atlantic salmon cell line derived from head kidney (SHK-1) was grown at 22 °C in an  
101 atmosphere of 4 % carbon dioxide in Dulbecco's modified eagle medium (DMEM) containing 3 g L<sup>-1</sup>  
102 D-glucose and 55 mg L<sup>-1</sup> sodium pyruvate, and supplemented with 10% foetal bovine serum (FBS),  
103 50 U mL<sup>-1</sup> penicillin, 50 µg mL<sup>-1</sup> streptomycin, 40 µM 2-mercaptoethanol and 4 mM L-glutamine. For  
104 gene promoter transactivation assays, fathead minnow (*Pimephales promelas*; FHM) epithelial cells  
105 were maintained at 22 °C in Leibovitz's L-15 with GlutaMAX<sup>TM</sup>-1 medium containing 900 mg L<sup>-1</sup> D+  
106 galactose and 550 mg L<sup>-1</sup> sodium pyruvate and 10% FBS. All media and supplements were obtained  
107 from Life Technologies (Glasgow, UK).

108 For subculturing, the cell monolayer was washed twice with phosphate buffer saline (PBS) without  
109 CaCl<sub>2</sub> or MgCl<sub>2</sub> (Invitrogen, UK), cells detached by incubation with 0.05 % trypsin/0.02 % EDTA  
110 and re-suspended in medium. Viable cells were counted after harvesting using a Neubauer  
111 haemocytometer, 0.4 % Trypan blue (Sigma, Dorset, UK) and an inverted microscope (IMT-2,  
112 Olympus).

113 For transcription factor (TF) ligand treatments, SHK-1 cells were seeded in 6-well clear plates (Nunc,  
114 Denmark) at a density of 4 x 10<sup>5</sup> cells per well in a volume of 3 ml Leibovitz's L-15 medium. Cells  
115 were approximately 70 % confluent after 48 h growth, when medium was aspirated, cells washed  
116 twice with PBS and fresh medium containing the various treatments as ethanol solutions was added.  
117 Final concentrations were cholesterol (20 µM), WY14643 (25 µM), 2-bromopalmitate (25 µM), or  
118 LXR agonists (GW3965 and T0901317, 10 µM) or with ethanol carrier alone (100% ethanol). After  
119 24 h, the medium was aspirated, the cell monolayer washed twice (PBS) and cells scraped from each  
120 well in 0.5 ml of PBS. Cells were centrifuged for 5 min at 3000 x g, PBS discarded and replaced by

0.5 ml of TriReagent (Ambion, UK), followed by vigorous mixing to lyse and digest cells. Cells from two wells were pooled to produce three replicates per treatment.

## *2.2. Fish, diets and sampling protocols*

Four diets (4 mm pellets) with the same basal protein composition, but coated with four different oils were formulated at Skretting Technology Centre (Stavanger, Norway) to satisfy the nutritional requirements of salmonid fish [14]. The oils used were FO (anchovy oil), or 100 % replacement with rapeseed oil (RO), linseed oil (LO) or soybean oil (SO). Atlantic salmon post-smolts (130 g) were randomly distributed into 16 tanks at the Skretting Aquaculture Research Centre (Stavanger, Norway). After a conditioning period of 3 weeks during which the fish received a commercial diet containing FO, the fish were fed the experimental diets to satiation for a period of 16 weeks. Full descriptions of the diet compositions and experimental conditions were reported previously [2]. At the end of the trial fish were anaesthetized with metacain (50 mg/L) and pyloric caeca (intestine), a major organ involved in uptake and transport of lipids, were dissected from five randomly selected fish from each dietary treatment. Samples of 0.5 g of caeca were immediately and rapidly disrupted in 5 ml of TriReagent (Ambion, UK) using an Ultra-Turrax homogenizer (Fisher Scientific, UK), and stored at - 80 °C prior to RNA extraction. The dietary trial and all procedures on Atlantic salmon conformed to European ethical regulations regarding the care and use of farmed animals in research.

## *2.3. Atlantic salmon LXR activation assay*

The ligand binding domain (LBD) (amino acid residues 191-462) [GenBank:FJ470290] of Lxr was amplified by PCR from salmon pyloric caeca cDNA using primers (Supplementary Table 1) with 5' restriction sites to allow in-frame subcloning between the *Bam*HI and *Xba*I sites of the pBIND vector (Promega, Southampton, UK), which contains the yeast GAL4 DNA-binding domain [8]. The resulting pBIND-Lxr chimeras were co-transfected with a reporter gene plasmid in which the *Firefly* luciferase gene is under the control of a promoter containing UAS (upstream activation sequence), which is recognized by Gal4. To control differences in transfection efficiency, a constitutively

expressed control reporter construct encoding for *Renilla* luciferase was included. Ligand activation of Lxr was determined by a luciferase-based functional assay using the FHM cell line as described previously [15]. Twenty-four hours prior to transfection,  $2 \times 10^4$  cells per well were seeded in a 96-well black-sided, clear-bottom microtitre plate (Corning, NY, USA). Transfection mixtures contained, per well; 100 ng pBIND-Lxr construct, 60 ng of luc2P/GAL4UAS reporter plasmid (pGL4.31, Promega), 20 ng of hRluc/CMV internal control reporter plasmid (pGL4.75, Promega) and 1.5  $\mu$ l of Polyfect transfection reagent (Qiagen) in 100  $\mu$ l of L-15 medium. Within each experiment, treatments were performed in triplicate. Experimental controls included treatments in which the pBIND-Lxr construct was replaced by empty pBIND vectors during transfection, as well as cells transfected with the appropriate pBIND-Lxr construct and reporters and treated with ethanol carrier only. After 24 h, transfection mixes were removed and replaced with media containing treatment vehicle (ethanol), cholesterol, or one of the following LXR agonists: natural oxysterols 20(S)-hydroxycholesterol (20S-OH) and 22(R)-hydroxycholesterol (22R-OH), synthetic agonists T0901317 and GW3965, and the fungal molecule paxilline (Sigma Aldrich, UK). Compounds of interest were diluted into L-15 from ethanolic stock and 100  $\mu$ l of the dilution was added per well and incubated for 24 h. Cells were lysed by 10 min incubation in 75  $\mu$ l per well of 1x Passive Lysis Buffer (Promega), and *Firefly* and *Renilla* luciferase activities quantified using an assay protocol described previously [15]. Transactivation activities were obtained using VICTOR X Multilabel plate reader (PerkinElmer, USA) and data were normalized to the *Renilla* luciferase activities. Data are presented as means of raw transactivation activities of three independent assays. The significance of effects of treatments were tested by one-way analysis of variance (ANOVA), followed by multiple comparisons using Tukey's *post hoc* test at a significance level of  $P \leq 0.05$  (PASWS 18.0, SPSS Inc., USA).

## 2.5. Quantitative RT-PCR (qPCR)

Salmon caecal samples from fish fed different oil sources ( $n = 5$ ), or SHK-1 cells exposed to Lxr- or Ppar-ligands ( $n = 3$ ) were used for relative and absolute qPCR analyses, respectively. For gene expression analysis, samples were immediately and rapidly disrupted in TriReagent, and stored at - 80



°C prior RNA extraction. Total RNA was extracted following the manufacturer's instructions (Ambion, UK), and the quantity and quality of isolated RNA determined by electrophoresis and spectrophotometry (Nanodrop 1000, Thermo Scientific, USA). One microgram of total RNA was reverse transcribed into cDNA using the Verso cDNA synthesis kit (Thermo Scientific, UK) and primed with random hexamers and oligo(dT) in a 3:1 molar ratio. The resulting cDNA was diluted 20-fold with nuclease-free water. For quantitative qPCR, oligonucleotide primers for target genes and housekeeping genes (*elf-1α* and *β-actin*) (Supplementary Table 1) were used at 0.3 μM with one-fortieth of the cDNA synthesis reaction (5 μl of a 1:20 dilution) and 10 μl of SYBR-green qPCR mix (ABgene, UK) in a total volume of 20 μl. Reactions were run in a Mastercycler RealPlex<sup>2</sup> (Eppendorf, UK). Amplifications were carried out including systematic negative controls containing no cDNA (NTC, no template control) and omitting reverse transcriptase enzyme (-RT) to check for DNA contamination. Thermal cycle and melting curves were performed as described previously with specific annealing temperatures for each primer pair shown in Supplementary Table 1 [16]. The qPCR product sizes were checked by agarose gel electrophoresis and the identity of random samples confirmed by sequencing. Absolute quantification was achieved by including a parallel set of reactions containing spectrophotometrically-determined standards consisting of serial dilutions of known copy numbers of linearised plasmid that contain the predicted amplification product for each measured gene. Results were expressed as mean normalized ratios (± SE) between the copy number of target genes and the mean copy number of the reference genes (*elf-1α* and *β-actin*). Differences in the expression of target genes among different treatments were determined by one-way analysis of variance (ANOVA), followed by multiple comparisons using Tukey's *post hoc* test (PASWS 18.0, SPSS Inc., USA). In contrast, the effects of diet on TF expression were analyzed for statistical significance using the relative expression software tool (REST-MCS, 2009 V2 [<http://www.gene-quantification.de/rest-2009.html>]), and normalized by two housekeeping genes (*elf-1α* and *β-actin*). Gene expression was presented as the relative expression ratio of each gene in fish fed one of the VOs or FO. A significance of  $P \leq 0.05$  was applied to all statistical tests performed.

## 2.6. Expression constructs and cell transfection assays

Atlantic salmon transcription factors Lxr [GenBank: FJ470290], Ppara [GenBank: AM230809], Ppar $\beta$ 1a [GenBank: AJ416953], Srebp1 [GenBank: HM561860] and Srebp2 [GenBank: HM561861NM\_004599] had been previously described [12,13]. The entire open reading frame of Lxr (Lxr ORF, 1-462 amino acids), Ppara (Ppara ORF, 1-464 aa), Ppar $\beta$ 1a (Ppar $\beta$  ORF, 1-443 aa), and the soluble N-terminal domains of Srebp1 (nSrebp1, 1-476 aa) and Srebp2 (nSrebp2, 1-460 aa) were amplified from caecal cDNA samples using the primers detailed in Supplementary Table 1, which included suitable restriction sites, *EcoRI* and *XhoI*, for subsequent insertion into the CMV-based constitutive expression vector pcDNA3 (Invitrogen, UK). Positive clones were selected by enzymatic digestion and sequenced (CEQ-8800 Beckman Coulter Inc., USA). The putative promoter regions, first non-coding exon, first intron and the ATG start codon of Atlantic salmon *elovl5a* (-3618, [GenBank:GU238431]), *elovl5b* (-3141, [GenBank:GU324549]), and *fads2d6a* (-1791, [GenBank:AY736067]) were amplified from genomic DNA using high-fidelity PfuTurbo DNA Polymerase (Statagene, UK) and primers containing restriction sites (Supplementary Table 1). These fragments were cloned into *SacI* and *NcoI* or *XhoI* sites encompassing the luciferase start codon of a promoterless reporter plasmid [pGL4.10, *luc2*] (Promega, USA), which encodes *Firefly* luciferase.

For luciferase assays, FHM cells were co-transfected, under the conditions described previously, with the salmon *elovl5* or *fads2d6a* reporter constructs, Lxr, Ppara, Ppar $\beta$ 1a, nSrebp1 or nSrebp2 expression constructs to overexpress the protein product. Briefly, stock FHM cells were seeded in 96-well opaque plates at a density of  $2 \times 10^4$  per well 24 h prior to transfection. Transfection mixtures consisted of 50 ng of pGL4.10 reporter construct (empty pGL4.10 vector in controls), 30 ng of pcDNA3 expression construct (empty pcDNA3 vector in controls), 20 ng of *Renilla* pGL4.75 and 1  $\mu$ l of Polyfect transfection reagent to 100  $\mu$ l of L-15 medium. Within each transfection experiment, each treatment was performed in triplicate. After 24 h, the medium was aspirated, monolayer washed twice with PBS, cells lysed, and *Firefly* and *Renilla* luciferase activities quantified as described previously [15]. Transactivation activities were obtained, data normalized and statistical analyses performed as described above.

### 3. Results

#### 3.1. Ligand specificity of Atlantic salmon LXR

Prior to testing for Lxr-dependent gene expression the ligand binding specificity of *S. salar* Lxr was determined. The salmon Lxr/Gal4 chimera was activated by synthetic Lxr ligands, including T0901317, GW3965 and paxilline, and also by the physiological oxysterols, 22(R)-OH and 20(S)-OH (Fig.1). The concentration-response curve for activation of Lxr indicated that T0901317 and GW3965 were the most potent agonists (Fig. 1). T0901317 and GW3965 activated salmon Lxr at micromolar concentrations with maximal effect observed at 10  $\mu$ M. The synthetic agonist paxilline exhibited the lowest effect, whereas cholesterol had no effect. No response was detected at 100  $\mu$ M for most agonists, perhaps due to toxic effects.

#### 3.2. Gene expression in SHK-1 cells

Salmon SHK-1 cells were incubated with Lxr (T091317 and GW3965) and Ppar (Wy14643 and 2-bromopalmitate) agonists to identify respective target genes. Incubation with synthetic Lxr ligands caused potent induction of *srebp* genes, with up to 9-fold increase for *srebp1* and 2-fold for *srebp2* (Fig. 2). Lxr agonists GW3965 and T091317 increased the expression of *fas* mRNA 3- and 6-fold, respectively (Fig. 3). Fatty acyl desaturases, *fads2d6a* (~3.3-fold), *fads2d6b* (~2.4-fold) and *fads2d5* (4-fold) mRNAs were increased by GW3965. Only *fads2d5* expression was affected by T091317 being increased < 2-fold (Fig. 3). The expression of mRNA for *abca1* transporter, which regulates cholesterol efflux, was strongly upregulated by both Lxr agonists, with ~14-fold increases (Fig. 3). In the presence of Ppar agonists, only *srebp2* expression responded, relatively weakly, to 2-bromopalmitate (Fig. 2), which is a dual Ppara and Ppar $\beta$  agonist. However, Wy1465, a specific Ppara agonist had no significant effect on *srebp2* expression. The expression of acyl-CoA oxidase (*acox*), HMG-CoA reductase (*hmgCoAR*) and fatty acid elongases, *elovl4*, *elovl5a* and *elovl5b* (Fig. 3) and *ppars* (Fig. 2) did not change significantly after incubation with Lxr or Ppar ligands.

### 3.3. Trans-regulation of genes related to LC-PUFA metabolism by Srebp, Lxr and Ppar

To investigate the regulatory role of Srebp, Lxr and Ppar on the expression of key genes of LC-PUFA biosynthesis, we established Atlantic salmon promoter luciferase assays in FHM cells. The Srebp transcriptional regulation of duplicated *elovl5a* and *elovl5b* elongases, and *fads2d6a* desaturase promoters was assessed by co-transfecting with the active nuclear DNA-binding region of either Srebp1 or Srebp2. Consistent with previous *in vitro* observations that *fads2d6a* contains an SRE response element in the promoter region [17] the data indicate that both Srebp1 and Srebp2 promote the expression of *fads2d6a*. The promoters of both *elovl5a* and *elovl5b* duplicated genes were also highly activated (Fig. 4) and Srebp2 showed higher activity than Srebp1 for *elovl5b* and *fads2d6a*, whereas *elovl5a* was activated equally by both Srebp mature proteins. Co-transfection of FHM cells with ligand-activated salmon Ppars did not stimulate expression from *elovl5a*, *elovl5b*, or *fads2d6a*; promoters, however the *fads2d6a* promoter was significantly activated by Lxr (Fig. 5).

### 3.4. Nutritional regulation of the expression of transcription factors

Regulation of TF genes in response to dietary lipid composition was examined in pyloric caeca from salmon post-smolts fed diets containing either FO rich in EPA, DHA and cholesterol, or VOs rich in C18 FA, 18:1n-9 (RO), 18:2n-6 (SO), or 18:3n-3 (LO) but lacking LC-PUFA and cholesterol [2]. Compared to the FO-fed group, there was a significant increase of *srebp1* and *srebp2* transcripts in the pyloric caeca of fish fed the RO and LO diets (Fig. 6). The same trend was observed in fish fed the SO diet suggesting biological significance although not statistically. No differences were found between the dietary groups with regard to the expression of *lxr* or *ppar*  $\alpha$ ,  $\beta$  or  $\gamma$ .

## 4. Discussion

Elucidating the regulation of lipid and fatty acid metabolism in fish at a fundamental level is critical to understanding the relationships between lipid biosynthesis and metabolism and dietary lipid supply in fish, and will be crucially important to sustain the growth of aquaculture against a background of

static or diminishing supplies of fish oil derived from wild fisheries. To address these relationships we have undertaken this study to investigate the role of key lipid-regulated TFs on several salmon genes in an Atlantic salmon cell line, SHK-1, with a focus on genes of the LC-PUFA biosynthetic pathway. The SHK-1 cell line was initially developed from salmon head kidney tissue and is possibly leucocyte-derived, showing both macrophage- or dendritic-like phenotypes [18]. However, as a suitable salmon liver- or intestine-like cell line is lacking, the SHK-1 cell line was recently successfully utilized as a model for studying salmon lipid metabolism [13] on the basis that the critical transcriptional regulators of macrophage lipid homeostasis were the same as those in liver and they regulated similar target genes [19,20]. Thus, the response of this cell line to supplementation or depletion of cholesterol or fatty acids suggested the involvement of Srebp and Lxr in regulating several critical lipid biosynthetic genes [13]. To extend these studies and investigate the roles of individual TFs and their target genes more specifically we first characterized the ligand-activation dependency of Lxr and developed promoter/reporter gene constructs to investigate the role of Srebp, Lxr and Ppar in Atlantic salmon.

#### *4.1. Lxr ligand activation*

A single Lxr subtype has been identified in various fish species (compared to two in mammals), and all show a highly conserved structure across teleost fish, amphibians, birds and mammals [8]. Although a salmon *lxr* cDNA had been characterized the response of the corresponding receptor to activating ligands had not been tested [12]. Accordingly, concentration-response curves for Lxr ligands indicated that natural oxysterols including 20S-OH and 22R-OH (but not cholesterol) and the fungal metabolite paxilline were activators of Atlantic salmon Lxr. As with other vertebrate LXRs [8], the synthetic agonists T0901317 and GW3965 were strong activators of Atlantic salmon Lxr. Thus the synthetic agonists T0901317 and GW3965 were considered suitable tools for cellular assays of Lxr function.

#### *4.2. Transcriptional regulation of genes of the lipid metabolism*

The genetic control of lipid metabolism involves a complex interplay of transcription factors and regulatory loops acting on many genes of lipid metabolism and transport. For example, in mammals, LXR can mediate the regulation of lipogenesis through the direct activation of genes involved in lipid biosynthesis, or in a SREBP1c-dependent manner [10,21,22]. In mammals, two SREBP1 isoforms, SREBP1a and SREBP1c, are encoded by a single gene through the use of alternative start transcription sites, while a separate gene encodes Srebp2 [23]. Given this complexity, elucidated from studies in human and mammalian models, the present study aimed to determine if similar regulatory systems exist in fish due to the increasing importance of understanding basic lipid metabolic processes in modern aquaculture. In agreement with recent studies performed in rainbow trout (*Oncorhynchus mykiss*) [24,25], treatment of Atlantic salmon SHK-1 cells with Lxr agonists resulted in the upregulation of mRNAs for several important lipid metabolic genes, including *fas* involved in *de novo* biosynthesis of fatty acids and *abca1*, which controls the reverse cholesterol efflux (See diagram in Fig. 7). Ligand-activated Lxr also induced the expression of both *srebp1* and *srebp2* in SHK-1 cells. Similarly, *SREBP1c* and also *FAS* and *ABCA1* are established direct LXR targets in various human and rodent systems [10,21,26]. However, the LXR-mediated induction of *Srebp2* is not observed in mammals [22], and may be specific to Atlantic salmon. According to our results, several groups have reported LXR autoregulatory behavior in mammals through binding to LXRE (LXR response element) present in LXR $\alpha$  promoter [26].

Although vertebrates have the capability for endogenous synthesis of monounsaturated fatty acids from the saturated fatty acid products of FAS through the action of stearoyl CoA desaturase (SCD or  $\Delta 9$  desaturase), they are incapable of creating LC-PUFA *de novo*. Vertebrates thus require either dietary LC-PUFA directly or, depending upon species, they can produce the physiologically important ARA, EPA and DHA by desaturation and elongation of dietary shorter chain PUFA, linoleic (18:2n-6) and  $\alpha$ -linolenic (18:3n-3) acids, through the action of Fads1 and 2 and Elovl5 [27]. Intense interest has been paid to vertebrate LC-PUFA enzymes given their hormonal, ontogenetic and/or nutritional regulation in vertebrates [28,29]. Salmon Elovl5 enzymes are encoded by two very similar duplicated genes [3]. Neither mRNA was affected by Lxr agonist in SHK-1 cells, despite the *elovl5* gene

promoters being activated by co-transfection with nSrebps, which indicates that, although Srebps were induced in the cell line, under the experimental condition used they were not processed to transcriptionally active forms. In mammals, *Elovl5* is also not an LXR target, but is secondarily activated through LXR effects on Srebp1c [30]. Salmon *fads2* was significantly increased by Lxr agonist *in vitro*, and strongly activated by nSrebps indicating that this gene is a direct target of both transcription factors. In mammals, LXR agonist increased *Fads1* and *Fads2* expression although the results were attributed to a secondary LXR-dependent induction of SREBP1c [31]. The promoter element of the *Fads2* gene conferring Srebp1 response is highly conserved in several vertebrates including Atlantic salmon, with a potential SRE site and a NF-Y cofactor site [17].

Notably, Atlantic salmon *elovl5* duplicates exhibit differential tissue expression patterns and respond differently to dietary nutrients [3,32]. Accordingly, our results show that *elovl5a* is equally regulated by both Srebp1 and Srebp2, whereas *elovl5b* regulation by Srebp2 is 2-fold greater than that exhibited in response to Srebp1, possibly as result of sequence divergence in the promoter regions of these genes.

PPARs are also central to the transcriptional control of lipid metabolism and several interactions with LXRs and SREBPs have been proposed [33,34]. Mice knockout assays and subsequent *in silico* analysis confirmed that PPAR $\alpha$  induces *Srebp1* and *Srebp2* expression through the interaction with PPRE (PPAR response element) in the mammalian *Srebp2* promoter [34]. In SHK-1 cells, few effects were observed upon treatment with WY14643, a Ppara specific ligand, or 2-bromopalmitate, a non-metabolizable fatty acid activator of teleost Ppara and Ppar $\beta$  [15]. However, bromopalmitate induced the expression of *srebp2*, whereas WY14643 had no effect. This suggests a Ppar $\beta$ -mediated effect, although it is also possible that the lack of response to the Ppara-specific WY14643 is due to a limited *ppar* subtype expression profile in SHK-1 cells.

#### 4.3. Dietary regulation of lipid transcription factors

The *in vivo* significance of the results from the SHK-1 cell line and promoter assays was previously shown by the observation that hepatic *srebp2* mRNA levels and LC-PUFA biosynthetic gene expression and biosynthesis increased in salmon fed diets containing high levels of VO [2]. In this study, similar results for the expression of LC-PUFA biosynthetic genes were observed in the intestine (pyloric caeca) of salmon fed VO [3]. The pyloric caeca of salmon constitutes the major tissue in terms of nutrient uptake, and lipid digestion and transport [35], and is also the tissue that exhibited the highest level of expression of *srebps* and *lxx* mRNAs in Atlantic salmon [13]. Therefore, if the LC-PUFA biosynthetic genes, *elovl5* and *fads2*, are driven by Srebps *in vivo*, then the same increase in Srebps should be observed in intestine from VO-fed fish. As the present study has shown, this was indeed the case. Our results indicated that, among the transcription factors studied, only *srebp1* and *srebp2* expression was increased significantly in the pyloric caeca of Atlantic salmon fed rapeseed oil (RO) and linseed oil (LO) compared to the expression in fish fed FO. Plant-derived products constitute the perfect activation ground for SREBPs. In the absence of sterols, the TF amino-terminus is proteolytically released from the endoplasmic reticulum membrane and transported into the nucleus where it binds SRE (serum response element) of specific sets of target genes [36]. In mammals, SREBP1 recognizes SRE sites in the promoters of *Srebp1* and *Srebp2* [37], thus it is likely that salmon Srebp1 activated in low sterol conditions, induced by the inclusion of VO, promoted its own expression and that of *srebp2* in a positive-feedback mechanism. Despite TF responses to dietary lipid variations in fish have been reported at specific developmental stages [12], no effects of VO inclusion were observed in the expression of *lxx* or *ppar* in this study.

## 5. Conclusions

The results of the present study showed that the fatty acyl elongases and desaturases responsible for endogenous production of LC-PUFA from PUFA in Atlantic salmon were primarily regulated by Srebps, and that Lxx may also be involved in regulating desaturases, but not elongases, whereas there was no evidence for a direct role of Ppars, at least in the salmon cell line tested. ABCA1, a gene central to the process of reverse cholesterol transport, and which is a direct LXR target in mammals,



was also a target of Lxr in salmon. Furthermore *fas* and both *srebp* genes, responsible for the major steps of lipogenesis, are direct Lxr targets in salmon as they are in mammals. Thus, overall, the transcriptional regulatory systems that drive cholesterol transport from the cellular space to lipoprotein, initiate lipogenesis, and regulate LC-PUFA biosynthesis in mammals are largely conserved in Atlantic salmon. This knowledge will be key to deriving a conceptual framework for future experiments designed to answer more applied questions related to lipid metabolism and nutrition with regard to the development and optimization of more sustainable aquaculture feeds. Importantly the present results also showed that these lipid regulatory factors and the genes that they target are ancient, likely to have arisen early in vertebrate evolution. Thus, further basic studies of evolutionarily conserved pathways of lipid metabolic control across vertebrates might also elucidate, through definition of fundamental regulatory mechanisms, medically relevant aspects of human lipid nutrition and metabolism.

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## Conflict of interest

The authors declare that there are no conflicts of interest.

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## Figure captions

### Figure 1. Activation of Atlantic salmon LXR.

Concentration-response curve for activation of Atlantic salmon LXR ligand-binding domain by T091317, GW3965, paxilline, cholesterol and oxysterols (20S-OH and 22R-OH). FHM cells were transfected with Gal4-LXR constructs, firefly luciferase reported plasmid pGL4.31 and an internal *Renilla* luciferase reporter to correct for transfection efficiencies. The ordinate represents activation of LXR as arbitrary units of firefly luciferase normalized to *Renilla* luciferase. Data points represent the mean of three independent experiments (n = 9).

### Figure 2. Effects LXR and PPAR agonists on transcription factor gene expression in Atlantic salmon SHK-1 cells.

Expression of *lxr*, *srebp1*, *srebp2*, *ppara*, *pparβ* and *pparγ* in SHK-1 cells exposed to 20 μM cholesterol (Chol), 10 μM of LXR agonists T0901317 (T0) or GW3965 (GW), 25 μM of PPAR agonists 2-bromopalmitate (2-BP) or WY14643 (WY), or carrier alone (EtOH). Gene expression is expressed as mean normalized ratios (n = 3, ± SE) between the copy numbers of target genes and the mean copy number of the reference genes (*elf-1α* and *β-actin*). Bars bearing different letters are significantly different (ANOVA; Tukey's test; *P* < 0.05).

### Figure 3. Effects LXR and PPAR agonists on lipid metabolic gene expression in Atlantic salmon SHK-1 cells.

Expression of Atlantic salmon lipid metabolic genes in SHK-1 cells exposed to 20 μM cholesterol (Chol), 10 μM of LXR agonists T0901317 (T0) or GW3965 (GW), 25 μM of PPAR agonists 2-

bromopalmitate (2-BP) or WY14643 (WY), or carrier alone (EtOH). Gene expression was expressed as mean normalized ratios ( $n = 3, \pm \text{SE}$ ) between the copy numbers of target genes and the mean copy number of the reference genes (*elf-1 $\alpha$*  and  *$\beta$ -actin*). Bars bearing different letters are significantly different (ANOVA; Tukey's test;  $P < 0.05$ ).

**Figure 4. Effects of SREBPs on LC-PUFA gene promoters.**

Co-transfection in FHM cells with *Srebp1*, or *Srebp2* expression constructs (empty pcDNA3 expression vector as control), and reporter pGL4.10 [*luc2*] directed by the promoters of salmon *elovl5a* (- 3618 nt), *elovl5b* (- 3141 nt), or *fads2d6a* (- 1791 nt). Error bars indicate SE between data points ( $n = 3$ ) of independent luciferase assays. \* Significant differences between treatments are indicated (ANOVA; Tukey's test;  $P < 0.05$ ).

**Figure 5. Effects of LXR and PPARs on LC-PUFA gene promoters.**

Co-transfection in FHM cells with *Ppara $\alpha$*  or *Ppara $\beta$*  expression constructs (empty pcDNA3 expression vector as control), and reporter pGL4.10 [*luc2*] directed by the promoters of salmon *elovl5a* (- 3618 nt), *elovl5b* (- 3141 nt), or *fads2d6a* (- 1791 nt). After transfection, FHM cells were incubated with *Ppara* agonists WY14643 (25  $\mu\text{M}$ ) or 2-bromopalmitate (2-BP, 25  $\mu\text{M}$ ), *Lxr* synthetic agonist GW3965 (10  $\mu\text{M}$ ), or ethanol carrier (EtOH). Error bars indicate SE between data points ( $n = 3$ ) of independent luciferase assays. \* Significant differences between treatments are indicated (ANOVA; Tukey's test;  $P < 0.05$ ).

**Figure 6. Nutritional regulation of transcription factor mRNA in Atlantic salmon intestine.**

*lxr*, *srebp1*, *srebp2*, *ppara*, *ppar $\beta$*  and *ppar $\gamma$*  mRNA expression in Atlantic salmon fed diets containing fish oil (FO), rapeseed oil (RO), soybean oil (SO), or linseed oil (LO) in the pyloric caeca determined by RT-qPCR. The results shown are means ( $n = 5$ )  $\pm$  SE of normalized expression ( *$\beta$ -actin* and elongation factor-1 $\alpha$  reference genes) in relative units (RU). Bars bearing different letters represent significant differences between dietary treatments for the respective transcripts (REST-MCS 2009 V2;  $P < 0.05$ ).

554 **Figure 7. Diagram indicating LXR and SREBP target genes.**

555 A) SHK-1 cells incubated with synthetic Lxr agonists (T0901317 and GW3965) showed that Lxr  
556 regulates multiple genes of the lipid biosynthetic pathway, mediating cross-regulation between  
557 cholesterol and fatty acid biosynthesis. Transcriptional expression of *srebp* was induced by Lxr  
558 agonist *in vitro* and dietary lipids (vegetable oils) in salmon intestine. B) Co-transfection assays in  
559 FHM cells indicated that Srebp1 and Srebp2 promote the transcription of *elovl5* and *fads2*, genes of  
560 the LC-PUFA biosynthesis; ER, endoplasmic reticulum; n, amino-terminus.



Figure1

Figure 1.

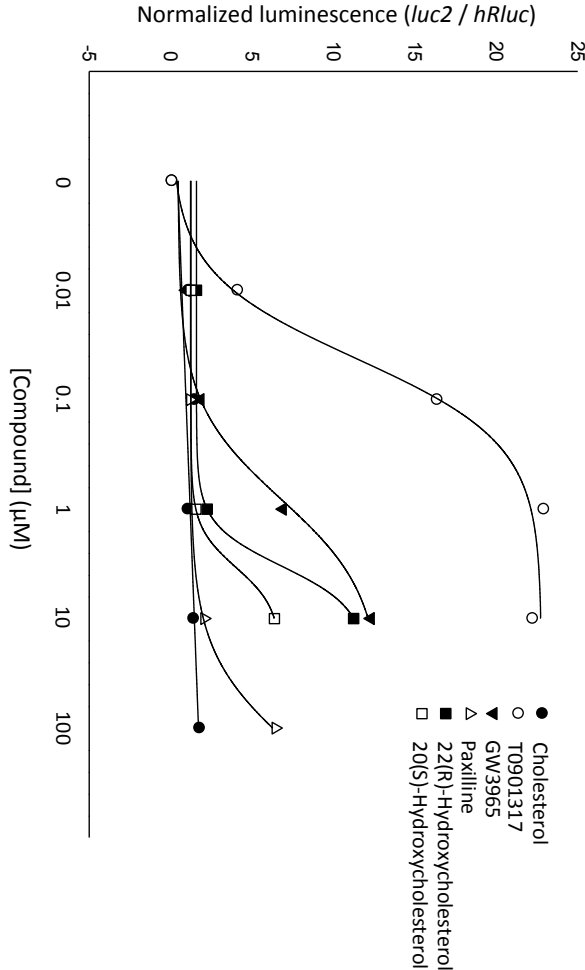


Figure2

Figure 2.

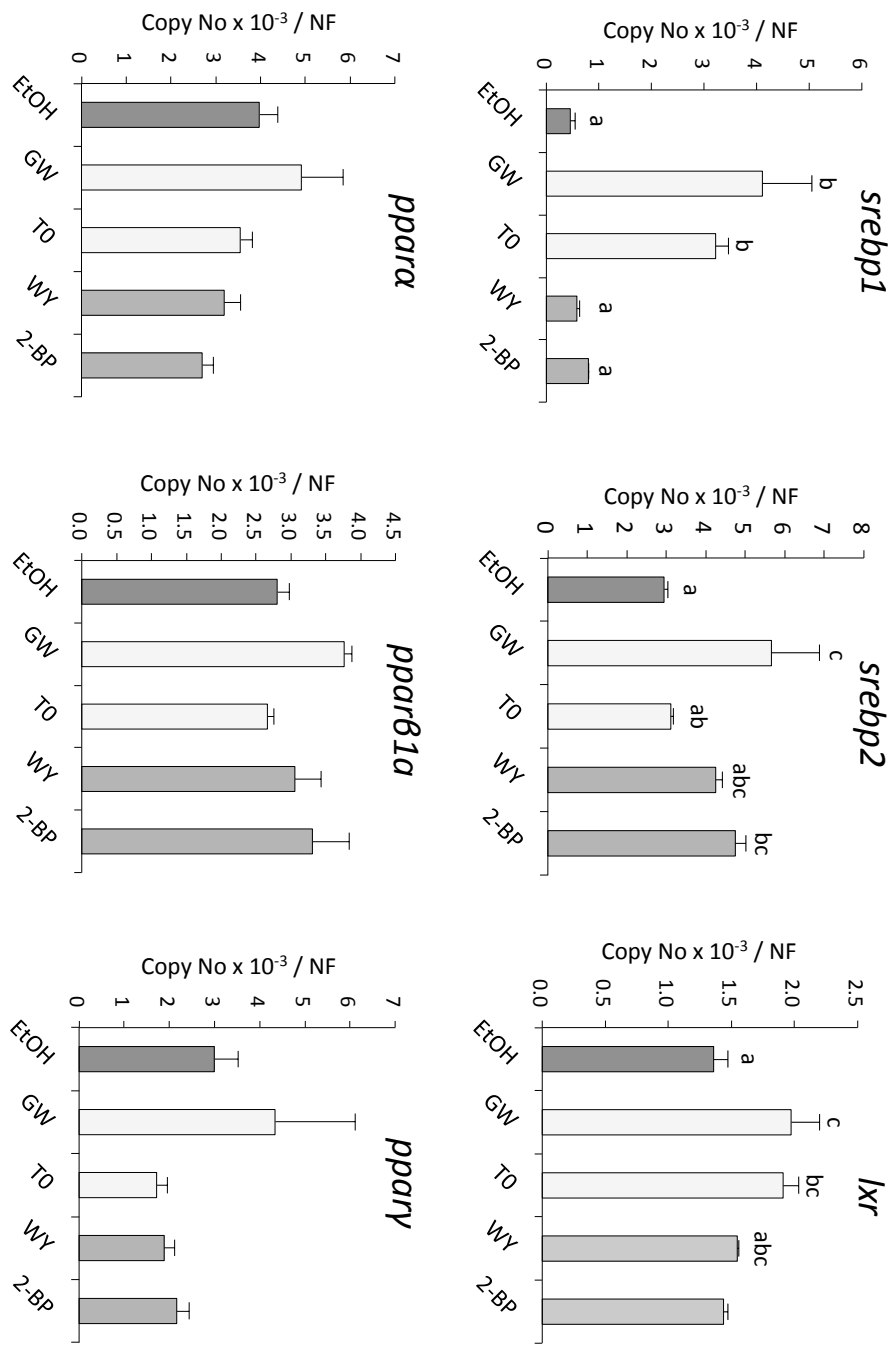


Figure3

Figure 3.

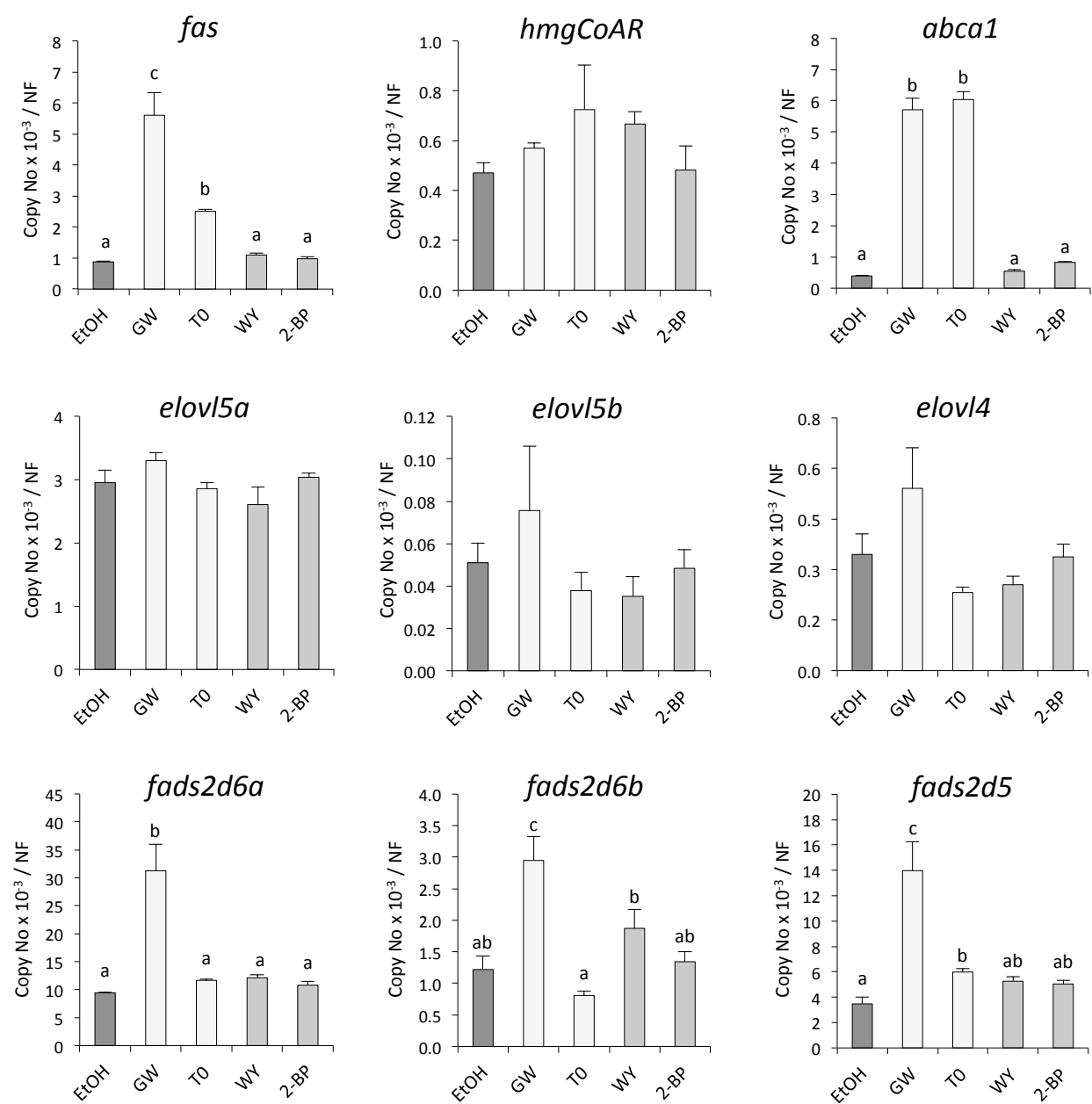


Figure 4.

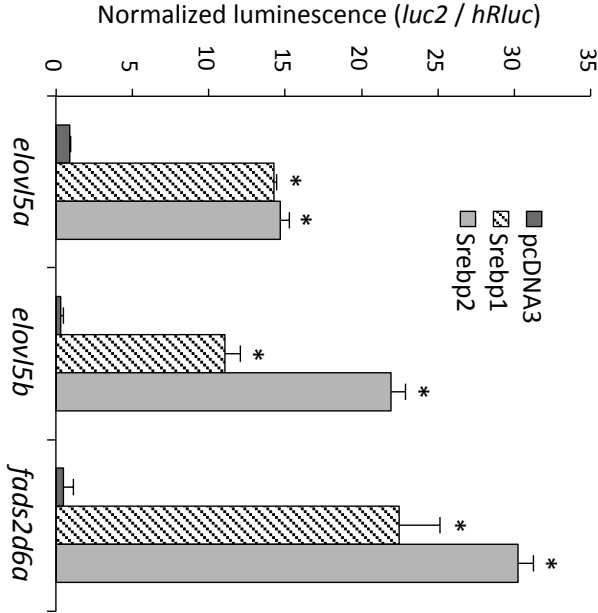


Figure 5.

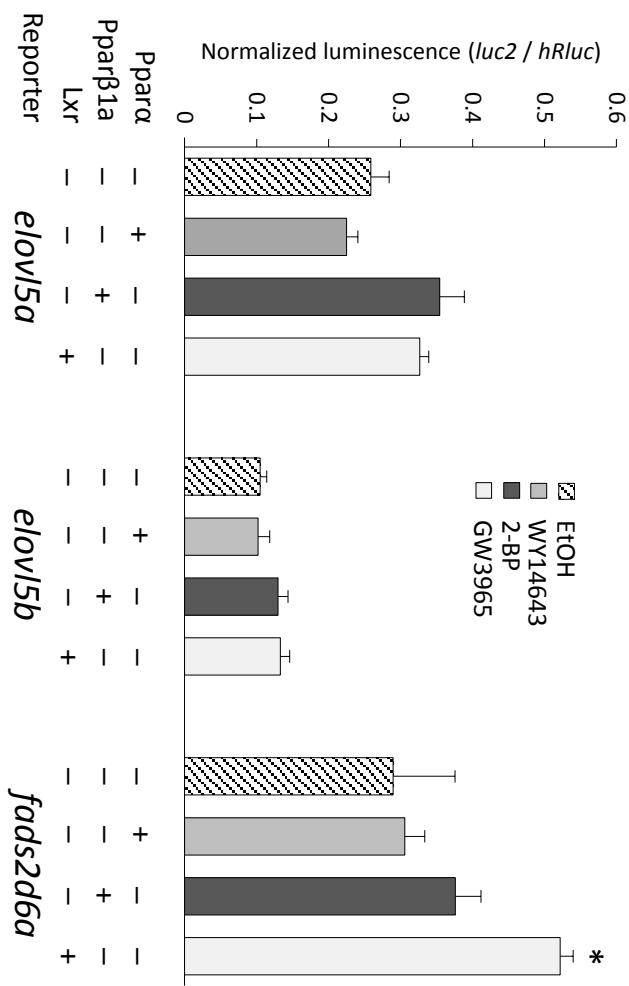


Figure6

Figure 6.

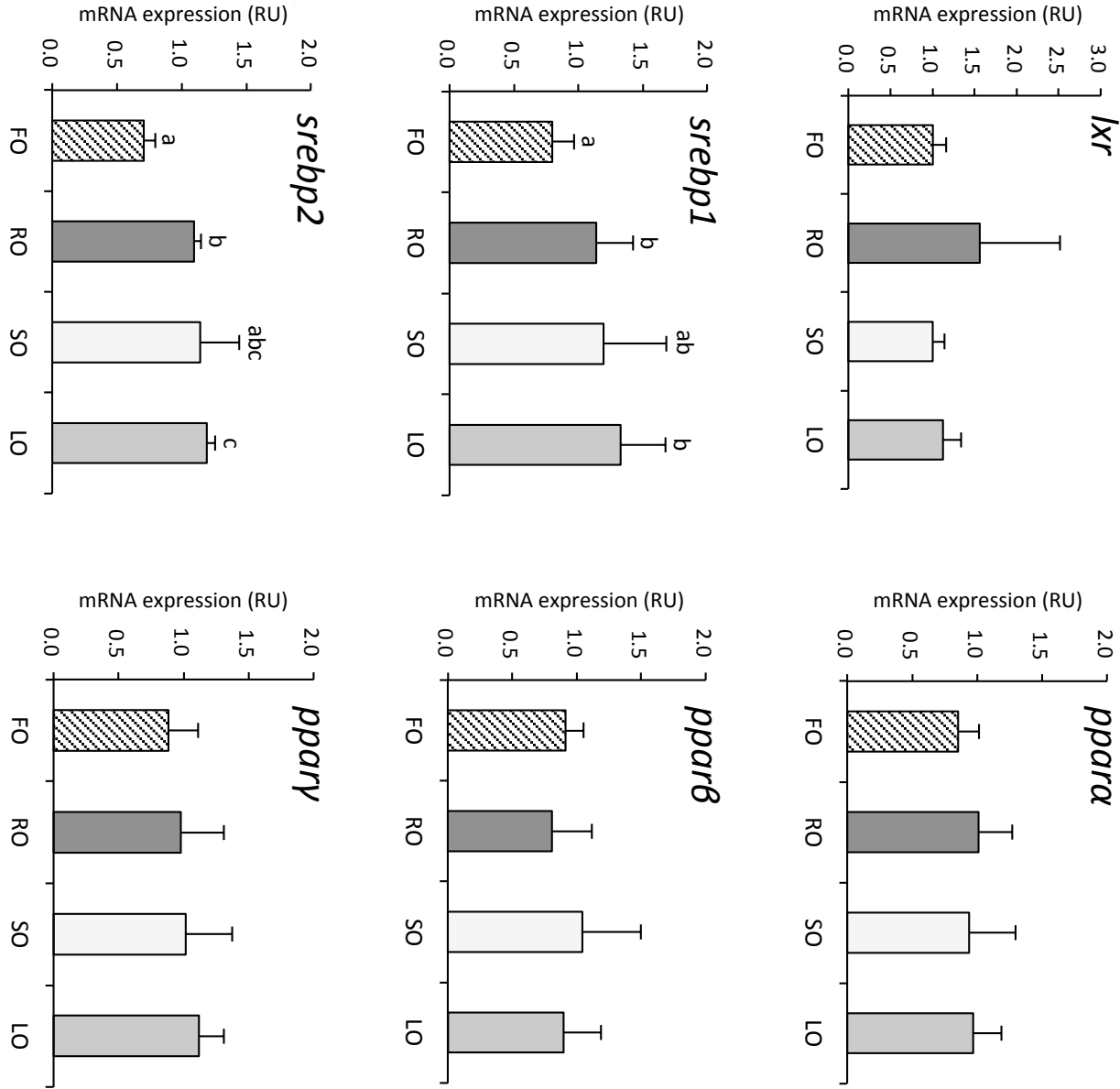
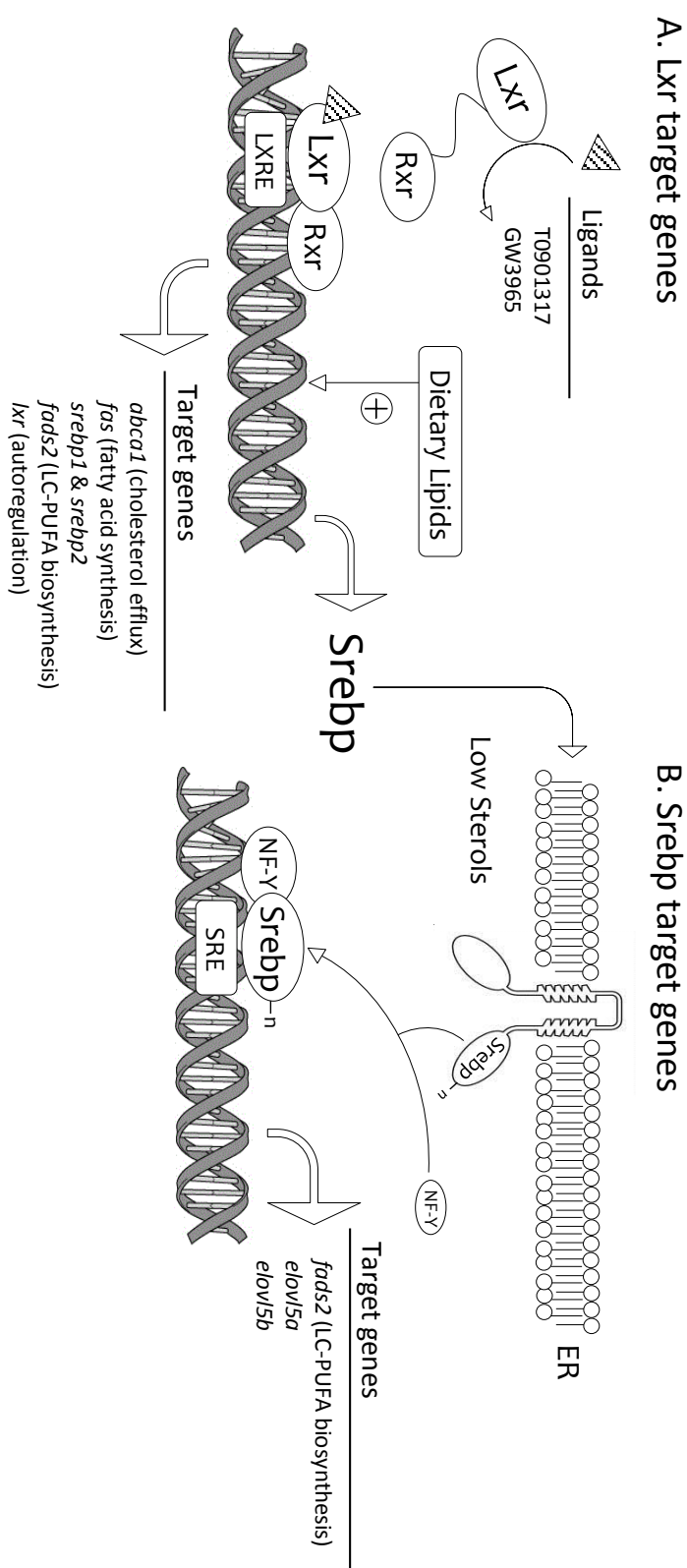


Figure 7.



**Supplementary table1**

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